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Temperature and pH Influence on Citric Acid Production from Orange Peels Using *Aspergillus niger*

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Abstract

Orange peels are undoubtedly abundant in nature, given the large volume of fruit juice produced daily across the globe from oranges coupled with ripe oranges consumed seasonally. Apart from juice industries, where they are obtained in cleaned form as raw materials for several products, including pectin and citric acid (CA), the alternative source is the uncleaned, often dry orange peels dumped in the surrounding environment. Arresting the environmental challenge posed by orange peels prompts its utilization in the grinded form to ferment and produce CA at different pH and temperature using 0.5 mL conidial suspension of *Aspergillus niger* in this study. At 5 days incubation period, Desing Expert 70.0 estimated 73.3% CA optimum yield at pH = 3 and optimal temperature of 70℃ and whose degree of substitution (DS) and reaction efficiency (RE) of 1.77 and 7.2%, respectively, implied that more citrate groups have been attached to each starch molecule. For increased emulsification, stability, and solubility, the CA produced at the highest DS and RE should be chosen to manufacture other products. Orange peel ripeness, moisture, micro or macro-nutrient contents, inoculum type, extraction method, and fermentation time (in the case of kinetic study) are influential factors to study during CA synthesis critically. Design of the solid-state fermentation technique used in this study at a laboratory scale is thus recommended.

Keywords: Citric acid, *Aspergillus niger*, Fermentation, Orange peel, Degree of substitution.

1 | Introduction

Citric acid (CA, $C_6H_8O_7$) or 2-hydroxy-1,2,3-propanetricarboxylic acid is a weak biodegradable organic tricarboxylic acid [1] consisting of 3 carboxyl (R-COOH) groups [2]. It is also a biochemical fermentation product of citrus fruits (or most plants) and animal tissues [3]. Extra pure anhydrous CA [4, 5] is very soluble

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and easily manufactured from molasses, carob pod extract, rape seed oil, corn-cobs, apple, lemon, orange, pine apple, plum, peas, peach, grape pomace, kiwi-fruit peel, mandarin orange, brewery wastes, animal bones, muscles and blood [6], and hence is contained in all fruit juices, soft drinks and syrups as it helps enhance their flavor and prevent color change by oxidation [7, 8]. Both laboratory and industrial manufacturing of CA followed the surface, submerged and solid-state fermentation production routes [1, 9] to recover CA for various industrial applications. Among them are fruits, dairy, cosmetic, food, chemical, beverage, textiles and pharmaceutical industries as a chelating agent, flavor enhancer, preservative, emulsifier, acidulant, antioxidant, preservative [6], pH regulator, stabilizer, digestion support, beverage sweetness balancing [9], synergistic agent, plasticizer [10], esterification agent for starch [11], buffering agent [12], toiletries, metal cleaners [13], organic coating material in the manufacture of nanoparticles [14] and for the modification of biomass for metal removal from wastewater [15].

These CA recovery fermentation techniques employ fungi, bacteria and yeast such as Saccharomycopsis lipolytica, Yarrowia lipolytica, Penicillium simplicissimum, Aspergillus foeitidus, Hansenula anamola, Candida tropicalis, Bacillus licheniformis, Arthrobacter paraffinens, Penicillium janthinelum, Mucor piriformis, Penicillium citrinum, Trichoderma viride, Ustulina vulgaris, Candida oleophila, Candida guilermondii, Bacillus subtilis, Brevibacterium flavum and *Aspergillus niger* [1, 6, 13, 16–18] by keeping track of one or more production-influencing factors shown in *Table 1*.

Table 1. Findings on citric acid production using *Aspergillus niger* and other organisms

Research	Biomass & Microorganism	Test & Optimal Condition	Method of Analysis	Citric Acid Yield
[19]	Corncob + *Aspergillus niger*	Temperature (23, 30, 37℃), fermentation time (0, 24, 48, 72, 96, 120, 144 h) & methanol conc. (0, 1, 2, 3, 4% v/w) Optimum – 72 h growth; 30℃; 3% methanol & 250 g/kg dry matter of corncobs	Statistica General Manova (StatSoft, Inc., Tulsa, OK)	> 50%
[3]	Banana peels + (*Aspergillus niger* & *Candida tropicalis*)	Optimum – pH 4; glucose 5% w/v; zinc 2% w/v; ammonium chloride 0.5% w/v & methanol 3% v/v for 10 days fermentation	Full Factorial Design RSM Design Expert	*Aspergillus niger*: 97.6 g/L at pH = 3.85 & Candida tropicalis: 113.6 g/L at pH = 3.45
[12]	Yam peels + *Aspergillus niger*	Nutrient medium (calcium ion, peptone, ethanol and manganese ion) Optimum values – Ethanol (7.0% v/v), manganese (1 g/L), calcium (0.1 g/L) and peptone (0.1 g/L)	Central Composite Design RSM (Multiple linear regression model) - Design Expert software version 7.0.0.	66 g/L
[6]	Red seaweed (*Gelidiella acerosa*) + *Aspergillus niger*	Control medium (pH = 1.5) Crude seaweed powder (pH = 3.5) Crude seaweed & 10% sucrose (pH = 3) Inoculation at 10 days, 200 rpm & 30℃	Experimental Study	80 g/L 30 g/L 50 g/L
[17]	*Parkia biglobosa* fruit pulp + *Aspergillus niger*	Production parameters – pH (2, 4, 6, 8, 10), inoculum size, substrate concentration (1, 2, 3, 4, 5%), incubation temperature (25, 35, 45, 55, 65℃) & fermentation period (5, 6, 7, 8 & 9 days)	Empirical Investigation	1.15 g/L at pH = 2; 0.53 g/L at 3% vegetative inoculum size; & 0.83 g/L at 2% substrate concentration

Table 1. Continued.

[20]	Cane molasses + *Aspergillus niger*	Maximum effect at 1 L/L/min oxygen tension, pH value of 6 & incubation temperature = 30℃. Reaction Condition: 15 L stirred fermenter (9 L working volume) with 150 g/L raw molasses sugars for 144 h	Laboratory Scale Fermenter	99.56 ± 3.5a g/L
[10]	Agricultural waste (wheat straw, rice straw & potato peel powder) + *Aspergillus niger*	25, 30, 35 & 40℃ temperature and 5.5, 6, 6.5, 7, 7.5 & 8 pH for 72 h incubation. Optimal values – 30℃ and pH = 6	Laboratory Experiment	0.38 g/100 mL
[21]	Olive mill wastewater + *Aspergillus niger*	The incubator running at 28℃ for 7 days	Aerated Two-stage Packed Column Bioreactor; Batch Fermentation	16 g/L
[22]	Banana & Plantain peels + *Aspergillus niger*	7 days fermentation; 60 g peels each; 27℃ incubation	ANOVA Statistical Analysis of Experimental Observations	0.172-1.304% from banana peels & 0.156-1.273% from plantain peels
[23]	Dates of date palm *Phoenix dactilifera* + *Aspergillus niger* strain	Effect of mould type and juice treatment Responses (mould kind) – total acidity (0.37-3.75 & 1.18-3.58%); final pH (3.74-2.57 & 2.9-2.58); final TSS (14-13.8 & 15.5-13.03)	Full Factorial Design with Minitab14	Percent acidity = > 3.5%
[2]	Sugarcane molasses byproduct + *Aspergillus niger*	Incubation for 144 h till 10 days at 28℃	Empirical Observation	9.6% with soil, 6.7% with air & 7.7% with bread`
[24]	Palm dates + *Aspergillus niger*	150 rpm mixing speed, 30℃ temperature, 11% sugar conc, 5% inoculum size & 7 days incubation		23.139% yield and 25.453 g/L concentration using date syrup
[25]	Spoiled coconut + *Aspergillus niger*	Optimum condition – pH = 3.5, temperature = 30℃, inoculum level = 5% & time course = 192 h	Simple Laboratory Run	82.65, 80.68, 83.24 & 85.18 g/L at the respective conditions

Aspergillus niger is the most favored microorganism for CA production, as showcased in *Table 1*, where various yields from a different selection of production conditions were achieved. Long before those studies, CA has been first isolated and crystallized by Swedish Chemist, Carl Wilhelm Scheele [25]. During its synthesis, factors including trace elements concentration, microorganism morphology, pH, concentration of alcohols, aeration, carbon, nitrogen and phosphorus sources are often examined [1, 13, 16]. CA's numerous applications warrant its large volume of manufacture on a very large progressive scale. In view of that, research efforts to optimize its production have been reported using design expert software *(Table 1)*. For instance, world CA production was around 1.4 million tons in 2004, increasing to 1.6 million tons in 2007 with an estimated present global yearly manufacture of about 736, 000 tons [13, 18, 25, 26] against 400, 000 tons earlier reported by Ali et al. [20]. De Medeiros & De Medeiros [27] mentioned that, about 2 million tons of CA are produced by submerged fermentation while 92% of all manufacture uses *Aspergillus niger*. In 2023, the world CA market was projected to clock at $3.2 billion [9, 16].

In anticipation of increased patronage of the useful acid beyond the present record, this study focuses on exploring a new or additional biowaste from which CA could be extracted. Specifically, powdered orange peels are experimented with using *Aspergillus niger* for CA content. Normally, the fungus, *Aspergillus niger*, thrives in decaying vegetation and soils [28], being the most commonly used microorganism for the extraction

of CA from plant wastes. Orange peels contain 42.5% pectin, 16.9% soluble sugars, 10.5% hemicellulose, and 9.21% cellulose [29], making them suitable carbon sources for CA production. The study aimed at being contributory, explicitly seeking to advance knowledge in former similar research conducted. Hence, Table 2 was deeply analyzed with the intention of addressing some identified drawbacks in those studies.

Table 2. Previous work on CA fermentation using orange peels and *Aspergillus niger* fungus

Research	Method	Material Content, Type or Property	Optimum Condition	CAY
[29]	Autohydrolysis	38.2 g/L free sugars, 8.3 g/L sucrose, 13.7 g/L glucose, 16.2 g/L fructose and metals (Ca, K, Mg & Zn)	130℃, 8 g/g liquid/solid ratio & 40 mL methanol/kg medium	9.2 g/L
[30]	SSF	Dried orange peels (zero moisture)	96 h incubation	0.42-1.04 g/L
[31]	Before & After Thermal Pretreatment	Press liquor from orange peel dewatering	5-6.2 pH & 40 mL/kg methanol	30g CA/kg press liquor & 730 g/kg due to treatment
[32]	Microbial Fermentation	21.43 g/L sucrose	30℃, pH 5 & 96 h incubation time	11.36 g/L
[33] & [34]	Ultrasound Assisted Extraction with RSM Optimization	20 mesh particle size dry orange peel	Range of conditions: 2-45 min, 50-500W ultrasound power & 0-100% ethanol (solvent) proportion	6.2g /100g dry orange peels
[35]	SSF	Dry and fresh peel	3 days fermentation	0.512% with methanol (dry peel) & 2.176% without methanol (fresh peel)
[36]	Submerged Fermentation	Oven dried and grounded peels	30±1℃, pH 6, 6-day incubation	0.63 mg
[37]	SSF	1-2mm size peel	30±1℃ & 3-5 days incubation	0.44%
[38]	Fermentation	11% sweet orange peel & 25% sucrose concentration	6 days fermentation, pH 4, 32℃, 2% inoculum	11 g/L
[39]	SSF	156.6, 137.3, 145.2 mg/g initial sucrose, glucose & fructose	30℃ incubation, 72 h fermentation	0.52g CA/g TS_c
[40]	Single & Co-culture Fermentation and RSM	60% moisture & 25g orange peels	6 days incubation, 6 mL inoculum size, 50℃ & 6 pH	94.92 mg/mL single & 114.68 mg/mL co-culture (A. niger + A. fumigatus) fermenation
Present Study	SSF	Size reduction of peel	70℃ & pH 3 with desirability of 92.41%	73.3%

CAY: Citric Acid Yield, RSM: Response Surface Methodology, SSF: Solid-state Fermentation, TS_c: Total Consumed Neutral Sugars

Plainly, among the three main CA fermentation routes, surface fermentation is hardly used in the production of the acid using *Aspergillus niger*. Few studies have looked at the effect of the sucrose, glucose, and fructose contents as well as the moisture content of the orange peels on the end product (*Table 2*). Effects of other physicochemical properties such as metallic nutrient content, total solids, ash content and particle density are hardly examined. However, almost all studies emphasized the importance of using low particle-size peels. New methodological approaches observed are the use of autohydrolysis and ultrasonic-assisted extraction techniques [29, 33, 34]. Furthermore, only one out of multiple microbes capable of extracting CA from orange peels is used on a few occasions, such as in Zafar et al. [40], where co-culture has been experimented. Most often, optimal pH, incubation time, methanol concentration and temperature during the synthesis are reported with little attention paid to the presence of inhibitors. Altogether (so far), less than 30 studies have

been conducted on CA production from orange peels based on the extent of this study's literature search, wherein yields of CA were reported in different dimensions.

As observed in Tables 1 and 2 of the literature review findings, RSM showcased an almost 'absence' from the records of concerned researchers on the subject matter. Hence, optimal values of influencing factors such as pH and temperature are analyzed using the Design Expert tool in this study, as the effect of higher temperature (> 65℃) is not tested on CA yield from orange peels. Several statistics based on specified input parameter ranges on the desired response, as well as its model and representative surface plots to determine the optimal parameters, are expected at the end of the study. Furthermore, the CA produced will be characterized by a degree of substitution and reaction efficiency to aid its choice for further application.

2 | Methodology

Extracting CA from orange peel using *Aspergillus niger* involves a fermentation process. Step-by-step laboratory procedures followed to actualized the designed objectives were also detailed.

2.1 | Materials and Equipment

A fermentation vessel, 50 mL conical flask, stirring equipment, centrifuge, CA assay kit, glassware (flask, & beakers), and autoclave for sterilization are some of the equipment needed for this study. Orange peels, *Aspergillus niger* culture, nutrient media (for *Aspergillus niger* growth) and sterile water were materials taken, which requires the use of one or more of the equipment listed.

2.2 | Experimental Procedure

2.2.1 | Screening of the fungal cultures

Aspergillus niger cultures were screened qualitatively for the production of CA, as described by Ali et al. [20]. Czapek-Dox agar medium (10 mL) was poured into individual sterile Petri plates and allowed to cool at room temperature. Approximately 0.5 mL of the conidial suspension of *Aspergillus niger* was transferred to each of the Petri plates. The plates were incubated at 50, 60 and 70℃ for 5 days. In the literature, the incubation period of 3-10 days at 23-65℃ had been practiced [10, 17, 19], with 30℃ being the most experimented temperature [6, 24, 25]. The plates were observed after incubation for yellow zones due to CA formation. Strains of *Aspergillus niger* with the widest yellow zone were used for further studies.

2.2.2 | Fermentation media

Basal medium was prepared by introducing orange peels (30 g) into 200 mL Erlenmeyer flasks in accordance with Chukwuemeka et al. [22]. The medium was supplemented with glucose and sucrose at 5, 10 & 15% w/v. The effect of nitrogen supplements was not studied, though 0.5% ammonium nitrate and 0.3% ammonium phosphate were added to the basal medium and moistened to 55% moisture content. This is because nitrogen sources constitute a limiting factor in CA production [6, 20]. Subsequently, the flask was cotton plugged and autoclaved at 121°C for 15 min, slightly in accordance with Amenaghawon et al. [12]. After cooling at room temperature, each medium was inoculated with the *Aspergillus niger* (6.0×10^6) suspension and incubated at different temperatures (50, 60 & 70°C) in a rotary shaking incubator for 5 days. Methanol (0–5%) was added to the flasks before fermentation. After fermentation, the medium was diluted with distilled water (1:4 w/v).

2.2.3 | Citric acid production

All equipment and media were sterilized to ensure aseptic conditions, which are crucial to preventing contamination. A polyethene bag size was used to collect orange peels obtained at Girei Market, Adamawa State, Nigeria. Before then, it was washed, dried, and ground using mortar and pestle. A prepared *Aspergillus niger* culture was prepared originally according to the instruction manual provided into a nutrient media. The grounded orange peels were added to a clean and sterilized fermentation vessel. Hang & Woodams [19] also ensured that their corn-cob biomass was cut into pieces before usage. Then, the *Aspergillus niger* culture was

added to the vessel containing the orange peels and mixed thoroughly. *Aspergillus niger* produces CA during its growth, and this can take several days; hence the fermentation conditions (pH, temperature and agitation) must be maintained. This research employs the method followed by Hang & Woodams [19] in the production of CA using corn-cob by *Aspergillus niger*.

2.3 | Optimization of Process Parameters

This work involves varying the pH (1, 2 & 3) and temperature (50, 60 & 70℃) for 13 separate runs in a vessel. Temperature and pH were taken as factors, while citric acid yield (CAY) was the response variable. In order to define their levels in Design Expert 70.0 for the purpose of optimization, a new response surface design (RSM) was created where the initial and final values or boundary values of the 2 factors were entered to check-in Central Composite Design (CCD). By default, the RSM tool takes 8 non-center points, 5 center points, rotatable alpha ($k < 6$) = 1.41421 and 1 replicate of factorial points for 13 runs shown in *Table 3*.

Table 3. Experimental Design by RSM-CCD Tool.

Run	Factors		Response
	Temperature (℃)	pH	Citric Acid Yield (%)
1	60	2	-
2	60	0.585786	-
3	60	2	-
4	50	1	--
5	60	2	-
6	60	2	-
7	45.85786	2	-
8	70	1	-
9	74.14214	2	-
10	60	2	-
11	70	3	-
12	60	3.414214	-
13	50	3	-

The Design-Expert software gives the possible combinations of Factor 1 (temperature) and Factor 2 (pH) to be experimented with to obtain a corresponding response value of the product (left blank). Once the fermentation process is complete, the fermented mixture in the vessels is harvested after 7 days. The centrifuge was then used to separate the solid residue (orange peels and mycelium of *Aspergillus niger*) from the liquid portion (containing CA). The solvent extraction method was then employed to separate CA from the liquid portion. After that, the concentration of the CA was determined analytically using a titrimetric approach by using 0.1 NaOH and phenolphthalein as an indicator and calculated as % according to Equation 1 and the method followed by Odu et al. [3], Subramaniyan et al. [41] and Bakhiet & Al-mokhtar [2].

$$\%\ \text{Citric Acid} = \frac{\text{Normality} \times \text{Volume of NaOH} \times \text{Equiv. wt. of CA} \times \text{Dilution Factor}}{\text{Weight of Sample (g)} \times 10\ \text{mL}} \quad (1)$$

Purified CA was later stored in appropriate containers. The calculated yields were recorded appropriately and entered into the Response tab in Design-Expert to obtain the ANOVA as well as the surface plots.

2.4 | Characterization

2.4.1 | Degree of substitution determination

The degree of substitution (DS) was determined or calculated using Equations 2 and 3 according to the method described in Ye et al. [11].

$$DS = \frac{162A}{100M - (M-1)A} \quad (2)$$

$$A = \frac{(V_0 - V_1) \times c \times M \times 100\%}{m} \quad (3)$$

Where A is the content of esterified carboxyl groups (%); M is the molar mass of the substituent (citrate: 175 g/mol); m is the mass of the samples (mg); c is the concentration of aqueous hydrochloric acid (HCl) solution (mol/L); V_0 is the volume of aqueous HCl solution consumed by the blank (mL); and, V_1 is the volume of aqueous HCl solution consumed by the esterified starch sample (mL).

2.4.2 | Reaction efficiency determination

On the other hand, the reaction efficiency (RE) was calculated using Equations 4 and 5 given by Ye et al. [11].

$$E = \left(\frac{DS}{\text{Theoretical DS}}\right) \times 100\% \quad (4)$$

$$\text{Theoretical DS} = \frac{C \times 162}{175} \quad (5)$$

Here, 'C' is the mass of CA (g) divided by the mass of dry starch (g), and 175 is the relative molecular mass of citrate anhydride, whose chemical formula is $Y(NO_3)_3.6H_2O$ [5].

3 | Results and Discussion

3.1 | Citric Acid Yield

It is obvious that the CAY span from 40-79% over 45.9-74.1℃ and 0.59-3.41 pH, as shown in Table 4. The effect of pH and temperature was previously studied by Maharani et al. [25].

Table 4. Citric Acid Percent Yield for RSM

Run	Factors		Response
	Temperature (℃)	pH	Citric Acid Yield (%)
1	60	2	60
2	60	0.585786	55
3	60	2	61
4	50	1	47
5	60	2	59
6	60	2	61.5
7	45.85786	2	72
8	70	1	40
9	74.14214	2	68
10	60	2	60.2
11	70	3	76
12	60	3.414214	79
13	50	3	60

Figure 1 now shows the variation of these two factors with the yield, in line with the *Table 4* result. The highest CAY of 79% corresponds to a pH of 3.4, showing that the production of the acid using *Aspergillus niger* occurs at a less acidic pH. A low CAY of 55% was observed when the pH = 0.59 and 40% & 47% at pH = 1. The inability of *Aspergillus niger* to thrive at low pH during the early stages of fermentation may be the cause of the lower CA generation at pH 1, as exemplified by Alhadithy & Yasin [24]. As for temperature, the optimal

value is 70℃ at 79% CAY. From these experimental deductions, it can be said that CA production is maximum at pH = 3.4 and temperature of 70℃. Future design of a CA manufacturing unit may consider the optimal conditions that will give a percentage of pH or greater. *Fig. 1* (a & b) shows that both pH and temperature are important reacting conditions in the production of CA as both are respectively sensitive and should be maintained at their peak.

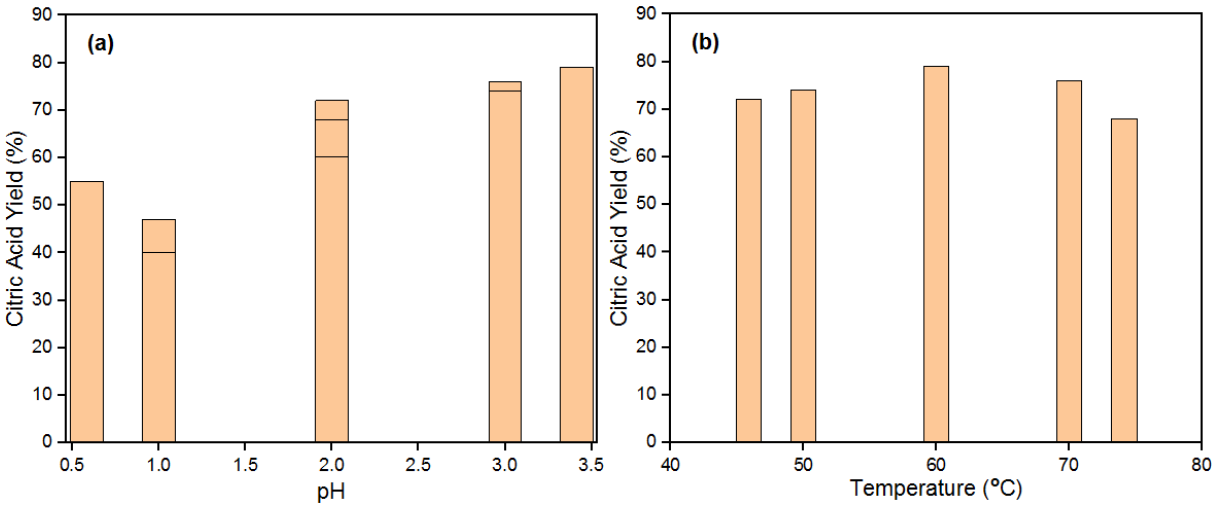

Figure 1. Effect of Varying Factors on CAY

Already observed is that the responses range from 40-79%, whose ratio of maximum to minimum is above 1.975. Normally, a ratio > 10 indicates a transformation is required. For ratios < 3, the power transforms have little effect. Since that is the case in this study, no transformation was selected for the purpose of this analysis.

3.2 | Statistical Analysis Summary

As explained in the methodology, the ranges defined by Factors A and B for 13 runs of quadratic modelling Based on CCD in the RSM software, also studied by Amenaghawon et al. [12], the 'Linear model' was suggested as the best model based on the predicted R^2 obtained in comparison with other model equations.

Table 5. Entry and specification analysis summary.

Design Summary:							
Study Type: Response Surface							
Runs: 13							
Initial Design: Central Composite							
Blocks: No Blocks							
Design Model: Quadratic							
Factor	**Name**	**Units**	**Type**	**Low Actual**	**High Actual**	**Mean**	**Std. Dev.**
A	Temp.	℃	Numeric	50	70	60	7.844645
B	pH	-	Numeric	1	3	2	0.784465
Response	**Name**	**Units**	**Obs.**	**Analysis**	**Minimum**	**Std. Dev.**	**Ratio**
CAY	R1	%	13	Polynomial	40	10.87353	1.975
*Suggested Model: Linear							

3.3 | Model Selection

At this juncture, a categoric warning was displayed by the Design-Expert software that the Cubic model is "Aliased" as shown in *Table 6*. Hence, it further instructed the selection of the highest-order polynomial where the additional terms are significant and the model is not aliased.

Table 6. Sequential model sum of squares.

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Mean vs Total	50806.253	1	50806.25	-	-	
Linear vs Mean	1188.8938	2	594.4469	17.07478	0.0006	Suggested
2FI vs Linear	20.25	1	20.25	0.555821	0.4750	
Quadratic vs 2FI	47.449923	2	23.72496	0.592187	0.5786	
Cubic vs Quadratic	105.60621	2	52.8031	1.510067	0.3069	Aliased
Residual	174.837	5	34.9674	-	-	
Total	52343.29	13	4026.407	-	-	

Even though the criteria of selection are well stated by the design tool, it still went ahead and pinpointed the 'Linear vs Mean' comparison as the best model for this study observations. Consistently, the selected model shown in *Table 7* must also have an insignificant lack of fit.

Table 7. Lack of fit tests.

Source	Sum of Squares	df	Mean Square	F-value	p-value Prob > F	
Linear	344.43113	6	57.40519	61.85904	0.0007	Suggested
2FI	324.18113	5	64.83623	69.86662	0.0006	
Quadratic	276.73121	3	92.24374	99.40058	0.0003	
Cubic	171.125	1	171.125	184.4019	0.0002	Aliased
Pure Error	3.712	4	0.928			

To this end, the best model remains the Linear Model as it has negligible lack-of-fit, as will be seen later. However, the most important statistical parameter RSM uses for model selection is the adjusted and predicted R^2. Their values are shown in *Table 8* for the different polynomial and factorial models under consideration.

Table 8. Model summary statistics.

Source	Std. Dev.	R^2	Adj. R^2	Prdct. R^2	PRESS	
Linear	5.9003655	0.773497	0.728197	0.51844	740.1757	Suggested
2FI	6.0359398	0.786672	0.715563	0.337649	1018.057	-
Quadratic	6.3295588	0.817543	0.687217	-0.28407	1973.666	-
Cubic	5.9133239	0.886251	0.727001	-6.12917	10957.8	Aliased

Therefore, the model maximizing the adjusted R^2 and the predicted R^2 was chosen, which is still the 'Linear' model. It has an adjusted $R^2 = 0.7282$ and a predicted $R^2 = 0.5184$, which is higher than its rival models, which are 2FI, quadratic and cubic models. Overwhelmingly, this study's observations (i.e., pH and temperature) can be set in a linear equation to predict the response or CAY equally.

3.4|ANOVA for Response Surface Linear Model

It consists of estimates of the sum of squares, degrees of freedom, mean square, F-value and the p-values of the model itself, parameters A & B, residual, lack of fit and the pure error obtainable, as showcased in *Table 9*.

Table 9. Linear Model ANOVA

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	1188.894	2	594.4468972	17.07478	0.0006	significant
A-Temperature	14.19607	1	14.19606781	0.407765	0.5375	
B-pH	1174.698	1	1174.697727	33.7418	0.0002	
Residual	348.1431	10	34.81431287			
Lack of Fit	344.4311	6	57.40518811	61.85904	0.0007	significant
Pure Error	3.712	4	0.928			
Cor Total	1537.037	12				

The Model F-value of 17.07 implies the model is significant. There is only a 0.06% chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, B is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve our model. The "Lack of Fit F-value" of 61.86 implies the Lack of Fit is significant. There is only a 0.07% chance that a "Lack of Fit F-value" this large could occur due to noise. A significant lack of fit is bad, as the fitted model is always desirable. The 3 R^2 statistical parameters for the linear model are shown in *Table 10*.

Table 10. Selected model statistical parameters computed by the design tool.

Statistical Measure	Value
Std. Dev.	5.900365
Mean	62.51538
C.V. %	9.438262
PRESS	740.1757
R^2	0.773497
Adj R^2	0.728197
Pred R^2	0.51844
Adeq. Precision	12.09192

The "Pred R^2" of 0.5184 is not as close to the "Adj R^2" of 0.7282 as one might normally expect. This may indicate a large block effect or a possible problem with the model and/or data. Things to consider are model reduction, response transformation, outliers, etc. Reports of Amenaghawon et al. [12] state that R^2 should at least be 0.8 for a model to be considered to be a good fit with observed data. In addition, "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. A ratio of 12.092 indicates an adequate signal. Such a model can be used to navigate the design space.

3.5 | Modeled Equation and Validation

In terms of A and B or temperature and pH, the CAY or R1 can be written as given by *Equation 6*.

$$\mathrm{CAY} = 46.27274 - 0.13321\mathrm{A} + 12.11764\mathrm{B} \quad (6)$$

Equation 6 results in predicted R1 or CAY shown in *Table 11*.

Table 11. Observed and predicted responses.

Factor A	Factor B	CAY (Expt.)	CAY (Prdct.)
60	2	60	62.51542
60	0.585786	55	45.37848
60	2	61	62.51542
50	1	47	51.72988
60	2	59	62.51542
60	2	61.5	62.51542
45.85786	2	72	64.39929
70	1	40	49.06568
74.14214	2	68	60.63155
60	2	60.2	62.51542
70	3	76	73.30096
60	3.414214	79	79.65236
50	3	74	75.96516

Some predicted CAYs are close enough to the empirical CAY, as evidenced in *Table 11*. However, despite the good statistical performance demonstrated by the linear model, considerable non-fit can be seen in *Fig. 2*.

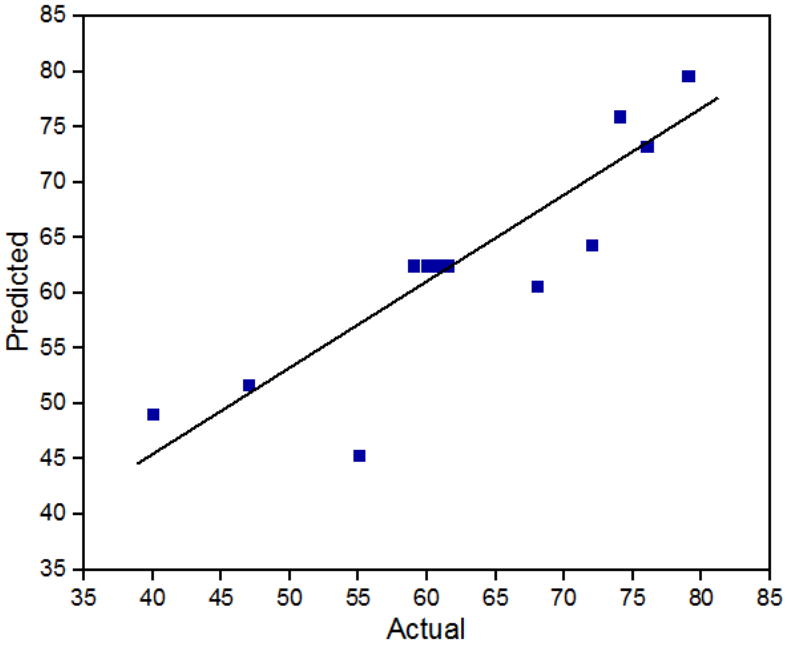

Fig. 2. Predicted CAY against actual CAY.

The slope of the fit is 0.7735, and the intercept is 14.16, which gives $R^2 = 0.773497$. Normally, an $R^2 = 1$ means a 100% fit, and a majority of studies try to keep the R^2 as close to 1 as possible. In this study, 22.65% of the participants were non-fit, which shows the extent of the fit.

3.6 | Citric Acid Optimization

Optimization of the CA production can be made to improve its manufacture using the microorganism selected. Figure 3 shows a surface plot of the highest desirability of the pH and temperature parameters/factors. It was obtained using Design Expert software, which is a statistical software package used for designing experiments, analyzing data, and optimizing processes. The surface plot in Figure 3 represents the relationship between pH and temperature and their effect on the response variable, which is the CAY. In a study conducted by Odu et al. [3], where the effect of pH & %methanol as well as pH & %nitrogen on CAY were examined using 3D surface plots, the representation reveals no interaction between nitrogen and pH but suggests that optimal CAY = 28 g/L occur at 2.6% methanol and 5.5 pH. Several other combinations

of factors had been examined in previous works, especially in Amenaghawon et al. [12], where nitrogen sources in the form of peptone demonstrated a significant effect on CAY. But here (differently), the plot shows that the highest desirability (0.924) of the pH and temperature parameters/factors is achieved at a pH of around 3 and a temperature of around 70°C. This indicates that these conditions are optimal for maximizing the CAY.

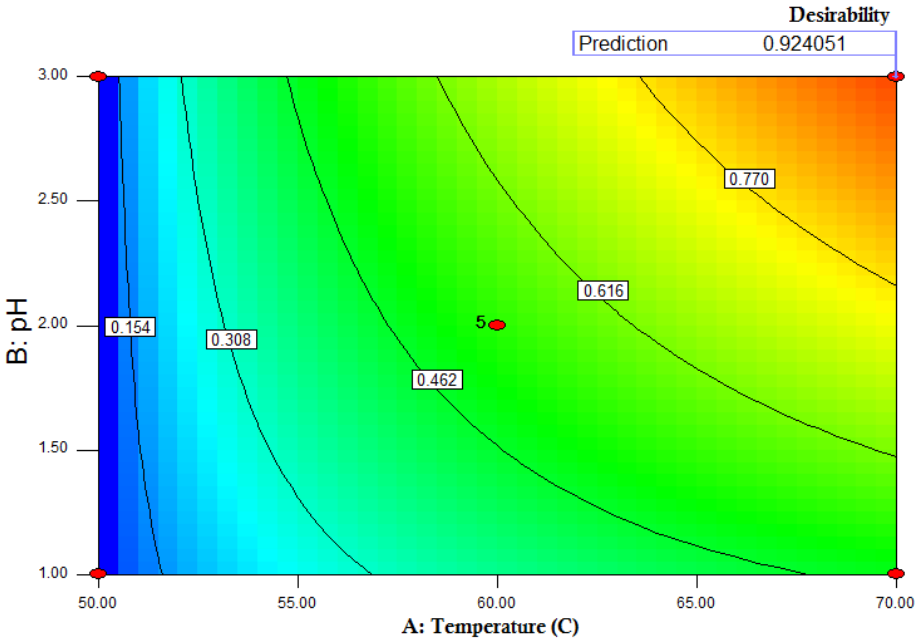

Fig. 3. Desirability of pH versus temperature optimization.

The surface plot also shows that the CAY decreases as the pH and temperature move away from the optimal values. This suggests that it is important to carefully control the pH and temperature during the fermentation process to achieve the highest possible CAY. Show et al. [1] reports that a previous study puts the pH value for CAY from *Aspergillus niger* at 2.5-3.5. By analyzing the surface plot in *Figure 4*, this study could identify the optimal conditions for the fermentation process and make informed decisions about how to adjust the process parameters to achieve the highest possible CAY.

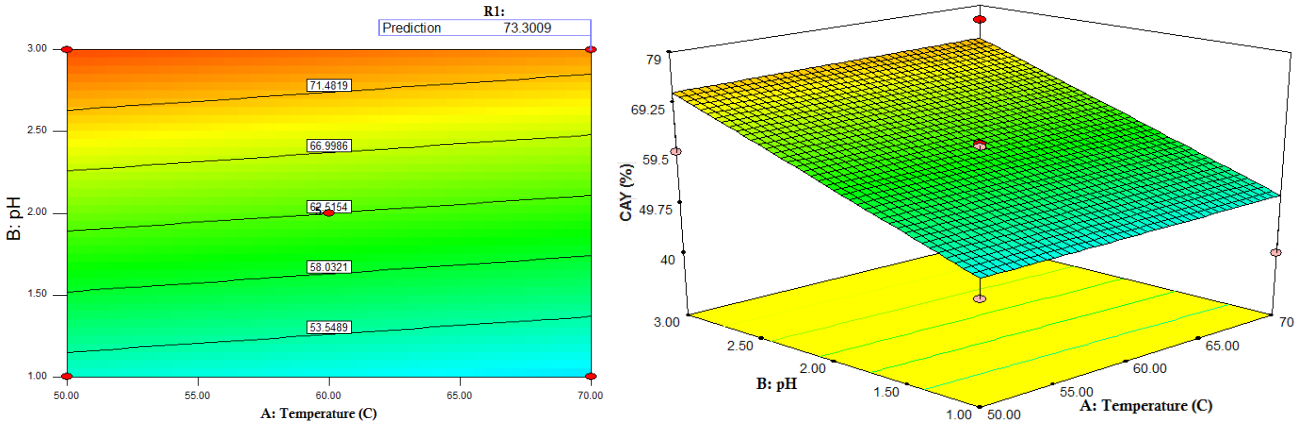

Fig. 4. Optimal predicted response at optimal pH and temperature.

Under the same desirability value, the maximal prediction for the CAY is 73.3009%, as shown in Figure 4. Variations in colors in the surface plots represent the magnitude of the response variable, which is the CAY in this case. In other words, the colors indicate the level of CAY at different combinations of pH and temperature. Typically, the colors in a surface plot range from cool colors (such as blue and green) to warm colors (such as yellow and red). Cool colors represent lower values of the response variable, while warm colors represent higher values. In the case of the CAY, blue and green colors would indicate lower yields, while

yellow and red colors would indicate higher yields. By analyzing the surface plot and the variations in colors, researchers can identify the optimal conditions for the fermentation process that result in the highest CAY. They can also identify the regions of the plot where the CAY is lower and make adjustments to the process parameters to improve the yield.

3.7 | Citric Acid Characteristics

Where M = 175 g/mol, a mass of CA = 30g, a mass of dry starch = 3.75%×30g = 1.125g, c = 26.67 and theoretical DS = 24.69, DS and RE were computed using relevant equations to obtain the values in Table 11.

Table 11. DS and RE determined for CA.

CA Conc. (%)	A (%)	DS	RE (%)
60	48	0.850022	3.442778
55	42	0.667582	2.703858
61	49	0.884555	3.582646
47	39	0.589696	2.388399
59	47	0.816778	3.308131
61.5	50	0.920455	3.728046
72	53	1.037207	4.20092
40	31	0.414836	1.680177
68	52	0.996687	4.036805
60.2	48	0.850022	3.442778
76	57	1.217884	4.932703
79	66	1.777261	7.198301
74	61	1.435086	5.812417

It is worthy of note that the main source of pectin is citrus peels, and pectin is classified through its degree of esterification (DE) or DS in order to define its gelling properties [42, 43]. In this case, Figure 5 was used to represent the DS and RE with the concentration of the acid measured experimentally.

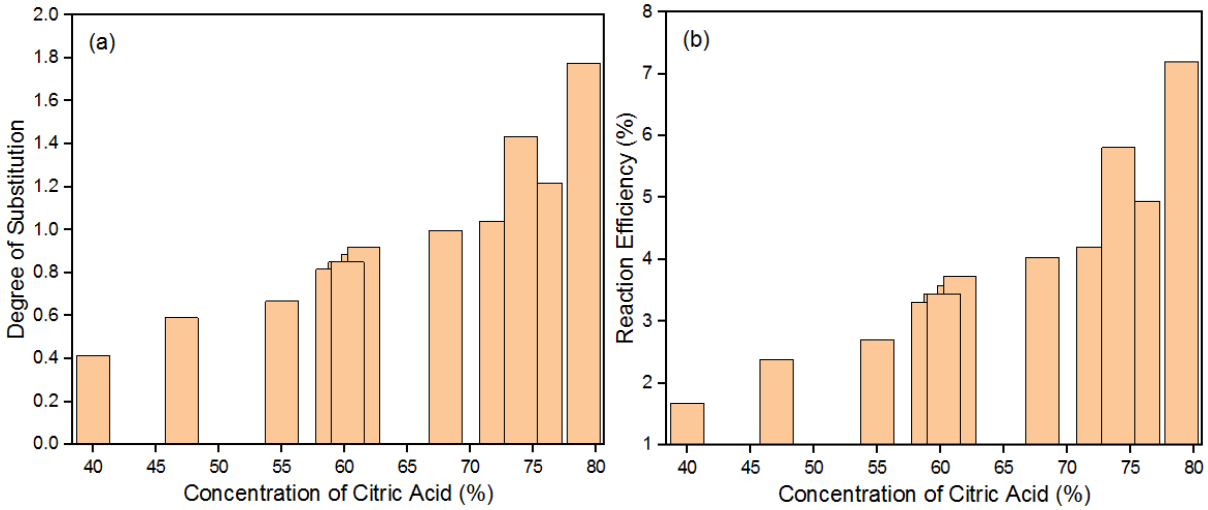

Fig. 5. Effect of CA Concentration on (a) DS and (b) RE.

DS is a parameter used to characterize the CA produced in the fermentation process. It is a measure of the degree to which the hydroxyl groups in the starch molecule have been replaced by citrate groups during the esterification process. In other words, DS is a measure of the average number of citrate groups that have been attached to each starch molecule. A higher DS value (e.g., 1.77 at 79% CA concentration) indicates that more citrate groups have been attached to the starch molecules, while a lower DS value (e.g., 0.41 at 40% CA concentration) indicates that fewer citrate groups have been attached. Citrate is used in the detergent industry

as a replacement for phosphate in detergents to make them more ecologically friendly [9]. RE is another parameter used to characterize the CA produced in the fermentation process.

It is a measure of the efficiency of the esterification reaction, which is the process by which the citrate groups are attached to the starch molecules. RE provides information on the effectiveness of the esterification process and the extent to which the citrate groups have been attached to the starch molecules. A higher RE value (7.2%) indicates that more citrate groups have been attached to the starch molecules, while a lower RE value (1.7%) shows that the esterification process was less efficient. Therefore, a CA product with higher DS and RE values may be preferred for applications that require properties like increased solubility, improved emulsification, and increased stability, as well as for applications that require a high-yield and high-quality product.

4 | Conclusion

There is growing application of orange peels in the extraction of CA using *Aspergillus niger*. This study successfully applied the SSF technique and varied temperature (50-70℃) and pH (1-3) to examine their influence on CAY, in addition to finding the optimal factors for the highest CAY in Design Expert 70.0. Findings in this study show that the acidic pH range (where pH = 3 is optimal) is the best for the grinded orange peels CA manufacture at a maximum approximate temperature of 70℃. Hence, optimal CAY was 79% observed and 73.3% predicted by the suggested linear model. Additionally, the produced CA may be suitable for high-yield product manufacture, especially at DS = 1.77 and RE = 7.2%, corresponding to the optimum pH and temperature. Adequate indication, both from the literature and the present study, emphasizes the effect of both pH and temperature in CA production from diverse materials, including orange peels. RSM 3D plot points to the leveled (equal) influence of the selected temperature range whose mean is 60℃, demonstrating that higher temperature is also favorable for the extraction. Since *Aspergillus niger* had been largely exploited, comparing the CAY from orange peels with several other microorganisms at specified or different inoculum sizes is encouraged.

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Author Contributaion

Conceptualization, Haruna Mavakumba Kefas; Methodology, Shamsiyyah Yakubu Saleh; Software, Abdulhalim Musa Abubakar; Validation, Cemre Avsar and Haruna Mavakumba Kefas; Formal analysis, Indianraj N and Abdulhalim Musa Abubakar; Investigation, Shamsiyyah Yakubu Saleh; Resources, Indianraj N and Cemre Avsar; Data maintenance, Haruna Mavakumba Kefas and Shamsiyyah Yakubu Saleh; Writing-creating the initial design, Abdulhalim Musa Abubakar and Cemre Avsar; Writing-reviewing and editing, Haruna Mavakumba Kefas and Indianraj N; Visualization, Indianraj N and Cemre Avsar; Monitoring, Abdulhalim Musa Abubakar and Haruna Mavakumba Kefas; Project management, Shamsiyyah Yakubu Saleh; Funding procurement, Shamsiyyah Yakubu Saleh and Indianraj N. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All the data are contained in this text.

Conflicts of Interest

The authors declare no conflict of interest. There aren't any personal circumstances or interests that could be considered to have an inappropriate influence on the presentation or interpretation of the reported research findings.

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